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The neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP) inhibits mitochondrial oxidative phosphorylation and replicates the pattern of neuronal neurodegeneration found in Parkinson's disease (PD). Neurons that degenerate in PD develop inclusions containing a synaptic protein, alpha synuclein. We examined how MPTP and other neurotoxins affect cytoskeletal and synaptic proteins and studied the relationship between oxidative damage and synuclein aggregation in MPTP-treated mice. Oxidative injury is evident in dopaminergic neurons 4 days after toxin administration followed 3-6 days later by increased synuclein and ubiquitin immunoreactivity. Double staining studies show that oxidative markers are increased in neurons that develop increased alpha synuclein staining. Paraquat treatment causes nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by MPTP. The proteasomal inhibitors lactacystin and epoxomicin protect mice from the neurotoxic effects of MPTP but may paradoxically increase alpha synuclein aggregation. Alpha synuclein knockout mice resist the neurotoxic effects of MPTP and other mitochondrial toxins including malonate, 3-nitropropionic acid and paraquat. Our studies show that MPTP-induced oxidative injury precedes increased neuronal alpha synuclein staining and suggest that alpha synuclein and abnormal proteasomal function could contribute to neurotoxin and PD-related neuronal cell death. Reducing synuclein expression may be a novel approach to the treatment of PD.

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Introduction

Intraneuronal inclusions called Lewy bodies, which contain the synaptic protein alpha synuclein, are a classical neuropathological hallmark of idiopathic Parkinson's disease (PD). Overexpression of mutant forms of alpha synuclein associated with familial PD can lead to aggregate formation in both transgenic mice (1) and fruit flies (2). The pattern of neurodegeneration found in Parkinson's disease (PD) can be replicated in some animal species, including primates and mice, by the systemic administration of neurotoxins such as 1-methyl-4-phenyl-tetrahydropyridine (MPTP)(3,4). MPTP inhibits mitochondrial oxidative phosphorylation and causes oxidative injury leading to cell death (3,5). Others and we have shown that MPTP can induce synuclein aggregation (1,4). Oxidative stress may be a key factor leading to synuclein aggregation that in turn may lead to further oxidative injury and the induction of neuronal death (6,7). The purpose of this study is to determine how MPTP and other toxins affect cytoskeletal and synaptic proteins and to study the relationship between oxidative damage and the formation of synuclein aggregates within neurons. In the first year of our study we showed that both acute and chronic MPTP treatment, which cause nigral dopaminergic neurons to degenerate, are associated with the displacement of alpha synuclein from its normal synaptic location into neuronal cell bodies. Neuronal degeneration was evident with DAT and calbindin immunocytochemistry and glial reaction was evident with GFAP immunocytochemistry. We also found that the redistribution of synuclein is associated with increased ubiquitin immunoreactivity and increased levels of oxidative markers in the substantia nigra and that the redistribution of synuclein does not appear to be associated with changes in distribution of synaptophysin or neurofilament proteins. In the second year of the study continued to make progress in accomplishing the experiments outlined in the Statement of Work by quantifying MPTP toxicity in three different strains of mice using two different protocols of MPTP administration. Synuclein aggregation was studied using four well-characterized alpha synuclein antibodies. In year three we made substantial progress in completing the proposed time course and double labeling studies proposed in the approved Statement

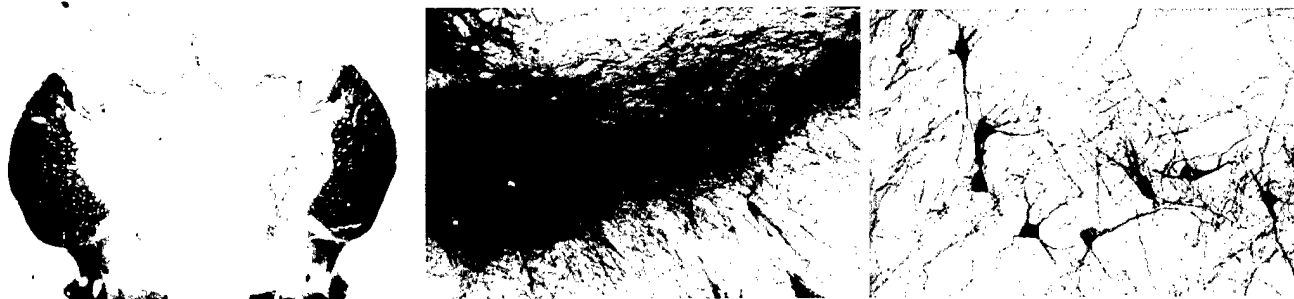
of Work and we pursued important new opportunities to study the role of synuclein in aggregate formation and neuronal injury that were not available when this grant was originally funded. We found that MPTP-induced oxidative injury is present in degenerating neurons prior to the development of increased synuclein and ubiquitin immunoreactivity. The role of synuclein in neuronal degeneration was directly examined in synuclein knockout mice and novel neurotoxins were tested that have the potential to be more potent than MPTP in inducing synuclein aggregation. We performed new studies using paraquat and the proteasomal inhibitor epoxomicin based on this important new data (8-11). In the final year of the study (year 4, no cost extension) we followed up on these new observations, completed data analysis and prepared a manuscript for submission incorporating the results of our studies.

Body

In the approved Statement of Work we proposed to complete two series of experiments in the three-year funding period. The purpose of the first series of experiments was to define changes in the distribution and morphology of alpha synuclein immunoreactivity produced by systemic treatment of MPTP in mice. Both the time course of these changes and their relationship to synaptic (synaptophysin) and neurofilament proteins (NF-M) were studied. The second series of experiments focuses on defining the spatial and temporal relationship between synuclein aggregation and oxidative injury at the cellular level. Patterns of cell death and apoptosis associated with MPTP toxicity were determined and related to the changes in synuclein and oxidative damage. Changes in the regional and cellular distribution of oxidative markers (3-nitrotyrosine, 8-hydroxydeoxyguanosine (8OHdG), and malondialdehyde (MDA)), DNA fragmentation (in situ end labeling, ISEL), and stress response (ubiquitin) with respect to synuclein in the substantia nigra and striatum of mice treated with MPTP were examined.

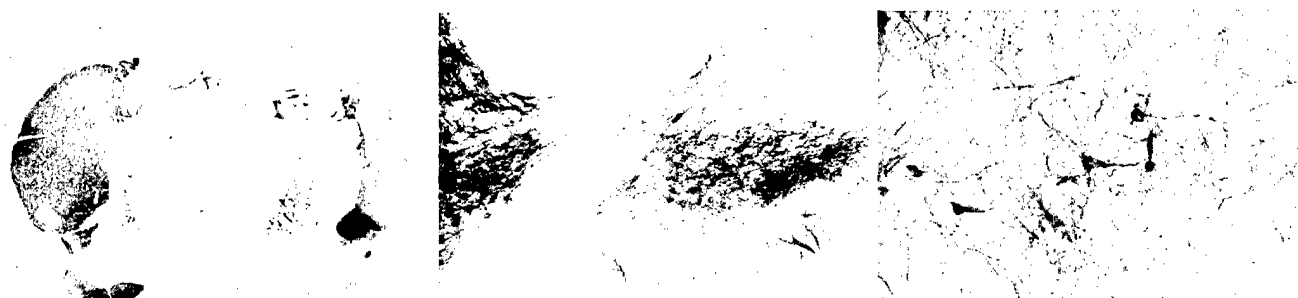
Two different MPTP toxicity models have been tested. Histological results are discussed herein and color photographs are included in the appendix materials. For the “acute” model 88 day old mice were given a single IP injection of 20 mg/kg MPTP every 2 hours until symptoms appeared. Animals were given five injections of MPTP on day one and four injections on day two, a total of 9 injections of MPTP. Control mice were given an equivalent volume of PBS according to the same schedule. The mice were perfused with paraformaldehyde 11 days after the last injection at a final age of 100 days. Serial sections of the brains were cut @ 50um into 8 wells. With this acute regimen, the mice were mildly symptomatic after the first day of five injections. It was not until the second day, at the time of the 8th injection that they were very symptomatic. For the “chronic” model, 88-day-old mice were given daily IP injections of 30 mg/kg MPTP. Control mice were given an equivalent volume of PBS at the same time (12PM daily). This regimen continued for 10 days. The mice were perfused 11 days after the last injection at the age of 108 days. Serial sections of their brains were also cut @ 50um into 8 wells. With this chronic regimen mice show little or no symptomatology for the first three days. On the fourth day, one hour after the injection, they became lethargic for 90 to 120 minutes. This behavioral response recurred daily after each injection. By the sixth day, the animals developed quickened respirations and hyperactivity immediately after the injection that lasted 15-20 min. This pattern of behavior also continued until the final injection.

The extent of MPTP-induced neurodegeneration was defined Immunocytochemically using a monoclonal antibody against the dopamine transporter (DAT). In our hands this has been a very reliable method to define dopaminergic neurons and their projections. The illustrations on the next page show a low power view of control, acute MPTP- and chronic MPTP-induced changes in DAT immunoreactivity in the striatum and the substantia nigra.



In the normal mouse striatum, DAT immunoreactivity is uniformly distributed (left, above). The substantia nigra (middle frame) is defined by a dense collection of DAT positive neurons. A higher power view of neurons in the caudal nigra (right, above) shows the extensive arborization of individual dendrites and axons in the control animals.

This picture is in striking contrast to the pattern of immunoreactivity in MPTP treated animals:



In the acute MPTP treated animal, there is a clear reduction in the intensity of immunoreactivity in the striatum (left, above). This depletion is more severe in the caudal and dorsal aspects of the striatum. The ventral striatum is less affected. There is also depletion of neurons in the substantia nigra, especially in the middle third of the nigra (A8 field) with relative sparing of the medial ventral tegmental area (A10) (middle frame). Higher power examination of individual DAT positive neurons shows dendritic and axonal pruning and fragmentation and distortion of immunoreactive processes (right, above).

Similar changes are seen in the chronic MPTP model but they are generally somewhat less severe, as shown on the next page.



The depletion of striatal DAT terminals is evident with relative sparing of the ventral striatum (left, above). This is accompanied by a depletion of DAT neurons in the nigra (middle, above) that is somewhat less severe than in the acute MPTP model. Similar morphological changes also affect dendritic and axonal processes (right, above).

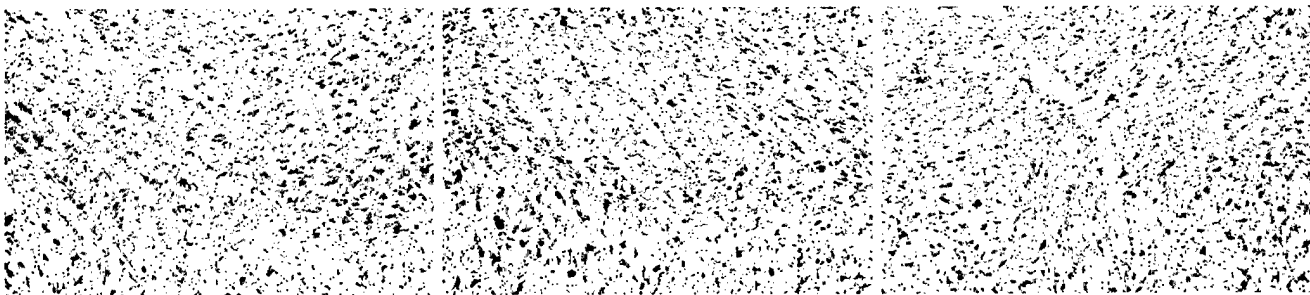
Quantification of cross-sectional area and density measurements of identified neurons were made using a Nikon Optiphot laboratory microscope configured with a "Cool-Snap", cooled CCD digital camera (Media Cybernetics, Silver Spring, MD). Video images were gathered and analysed using "Image Pro Plus" image analysis software (Media Cybernetics, Silver Spring, MD) running on a Dell Precision Workstation. Statistical analyses (ANOVA, t-test and Mann-Whitney U test) of data were performed using Excel (Microsoft, Inc, Redmond, W) and "Prophet" (NIH, NCRR and BBN Systems and Technology, Cambridge, MA).

Neuron cross-section measurements were made using spatially calibrated digital photomicroscopic images of the substantia nigra taken at 10 X and stored on the computer hard drive. Using the image analysis software, a mouse is used to trace the perimeter of niagral cell bodies that were stained by DAT. Only cells containing a nuclear ghost were measured and the circumference at dendrites was taken as a tangent across the base of the process. Measurement data were then exported directly to Excel for statistical evaluation.

The Integrated Optical Density of DAT terminal staining in the caudate was calculated using spatially calibrated digital photomicroscopic images of the striatum were taken at 1 X and a sample area (AOI) of 21030 μm^2 was used to measure the integrated optical density of MPTP lesions. Two reading from several sections from each animal were made by measuring the optical density of DAT

staining in a striatal lesion and then at a nearby unaffected striatum. To normalize the data, the measurements were expressed as a ration of the unaffected tissue measurement divided by the value of the lesion. Similarly, mean optical density was calculated on spatially calibrated digital photomicroscopic images of the striatum were taken at 1 X. The software was used to trace the perimeter of DAT stained striata from three sections in each animal centered around the level of the anterior commissure. The mean optical density (MOD) of each section was then divided by the MOD of the corpus callosum. Results are expressed as the mean \pm SEM. Statistical comparisons were made using one-way ANOVA followed by Fisher's PLSD *post hoc* tests.

The lesions induced by MPTP are also evident in cresyl violet stained sections of the substantia nigra (below). In the normal substantia nigra neurons are clearly visible (left, below). Neuronal depletion and glial increase is seen in both the acute MPTP (middle, below) and chronic MPTP (right, below) models.



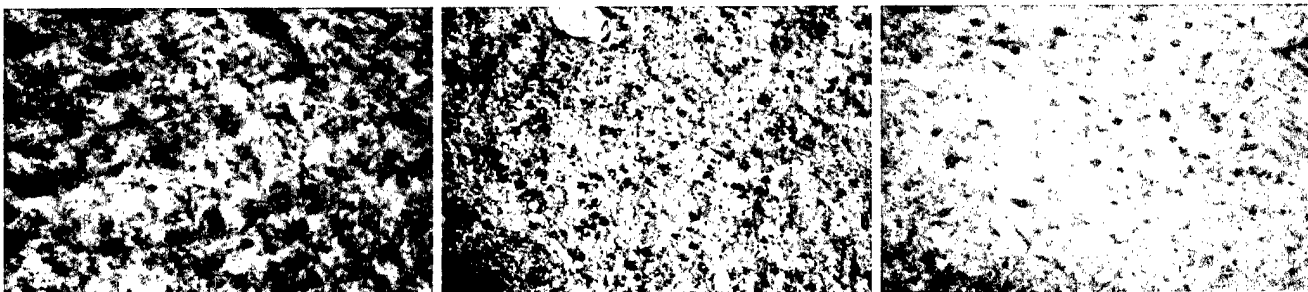
Quantitative studies were performed on neurons identified using DAT immunocytochemistry because it is the best approach to assess damage specifically affecting the dopaminergic neurons of interest. Samples of original data and analysis including statistics are provided in the attached appendix materials.



Glial fibrillary acidic protein (GFAP) immunocytochemistry (above) discloses astrocytes in brain tissue. In the acute (middle, above) and chronic (right, above) MPTP lesioned nigra the number and intensity of GFAP-positive astrocytes is clearly increased.

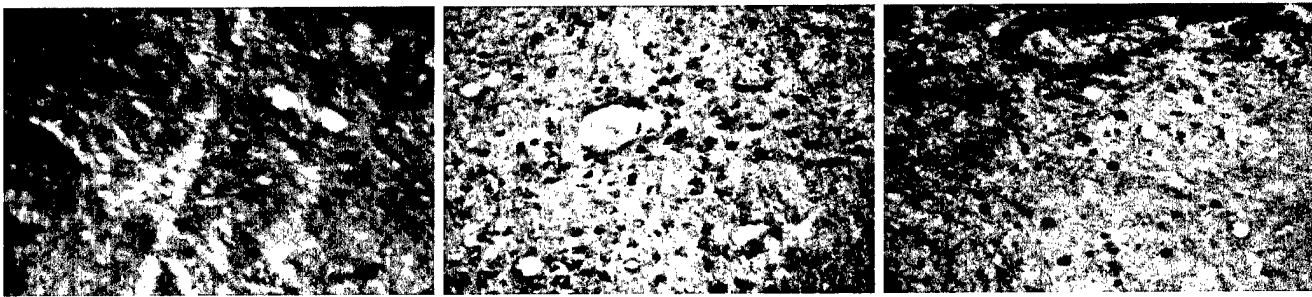
In the following series of photographs, changes in the immunocytochemical localization of relevant biomarkers in the substantia nigra of representative animals lesioned with the acute and the chronic MPTP protocol are depicted.

Calbindin is a calcium binding protein enriched in a subset of neurons in the substantia nigra. In the normal substantia nigra immunoreactive neurons are defined (left, below). In both the acute and chronic MPTP lesioned animals there is striking depletion of calbindin immunoreactivity (below, middle and right panels).

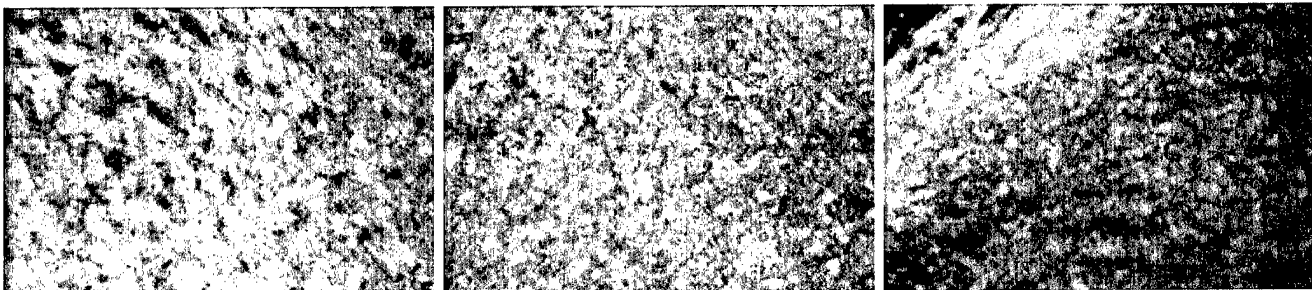


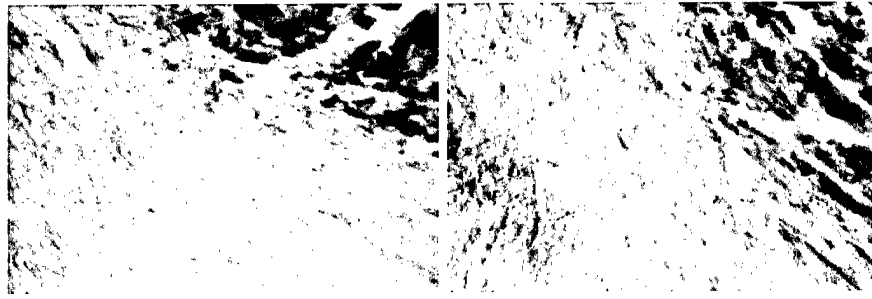
Alpha synuclein immunoreactivity in the substantia nigra of the normal mouse is punctate and resembles synapses (left above). The loss of calbindin and DAT immunoreactive neurons is associated with increased synuclein immunoreactivity in both the acute (middle, above) and chronic (right above) MPTP models.

Similar changes are seen with ubiquitin immunocytochemistry (below). A few ubiquitin positive cellular profiles are seen in the control substantia nigra (below, left). In the acute and chronic MPTP lesions (below, middle and right panels respectively) there is a clear increase in the number of ubiquitin positive profiles.



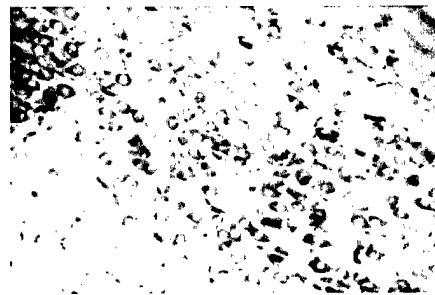
In contrast to the striking changes seen with alpha synuclein and ubiquitin staining, the staining pattern of synaptophysin, a synaptic protein, and neurofilament (medium chain), a marker of cell bodies and dendrites, changes minimally (below).





Neurofilament (in control, left above, and acute MPTP model, right above)

A series of 40 MPTP-treated mice were studied for evidence of oxidative injury. Markers of oxidative damage, such as 8-hydroxydeoxyguanosine, a marker of DNA oxidation, were clearly increased in neurons in the substantia nigra of MPTP-treated animals at 4, 7, and 10 day time points post MPTP injection (below).



Because in situ methods to detect DNA fragmentation have been shown to not be specific for apoptosis, we used a novel immunocytochemical marker to detect apoptosis: activated caspase 3 antibody (Idun). As shown in the appendix, increased immunoreactivity is detected in the substantia nigra of MPTP treated mice, consistent with activation of apoptotic pathways.

The severity of nigral damage produced by MPTP differed among different mouse strains. C57BL6 animals were more resistant to the effects of MPTP toxicity than B6CBA mice. Lesion size was smaller and more variable. Quantitation of MPTP toxicity in B6SJL mice, however, showed a greater mean reduction in DAT-positive neurons and less variability within individual groups. Four anti-alpha synuclein antibodies were tested (Zymed, Chemicon, Affinity, and courtesy of D. Clayton). Quantitative analysis using all antibodies showed an increase in synuclein positive cell bodies after MPTP treatment. Similar changes are seen with ubiquitin immunocytochemistry. A few ubiquitin positive cellular profiles are seen in the control substantia nigra. In the acute and chronic

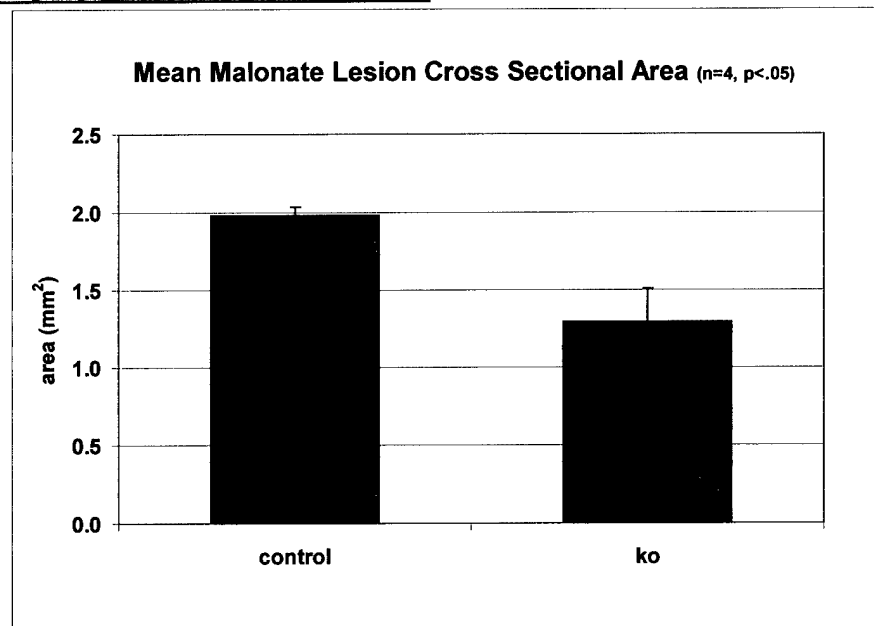
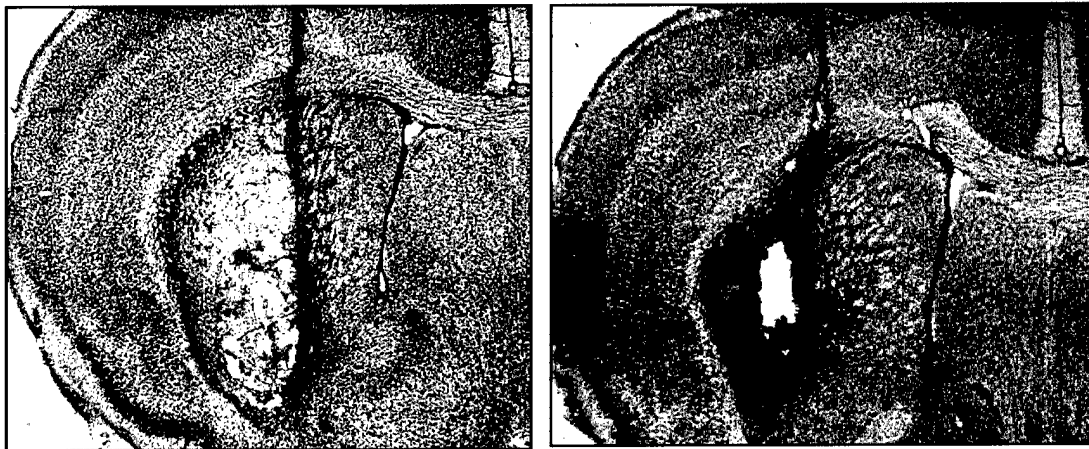
MPTP lesions there is a clear increase in the number of ubiquitin positive profiles. In contrast to the changes seen with alpha synuclein and ubiquitin staining, the staining pattern of synaptophysin, a synaptic protein, and neurofilament (medium chain), a marker of cell bodies and dendrites, changes minimally, as shown above.

Since both protocols resulted in similar lesions, the acute model was used in year 3 studies. We performed double labeling studies as proposed in the statement of work. We found that the oxidative markers colocalized in neurons with increased synuclein formation 7-10 post MPTP treatment. Histological studies of the substantia nigra 24 hours after the last MPTP injection showed no qualitative histological differences from controls. Synuclein, ubiquitin and oxidative marker (8 OHDG, 3NT, MDA) staining were normal. Oxidative markers were increased 4 days after the last MPTP injection but synuclein and ubiquitin staining were normal and neuronal loss was not evident despite clear cell loss and increased synuclein and ubiquitin immunoreactivity 7-10 days post MPTP treatment.

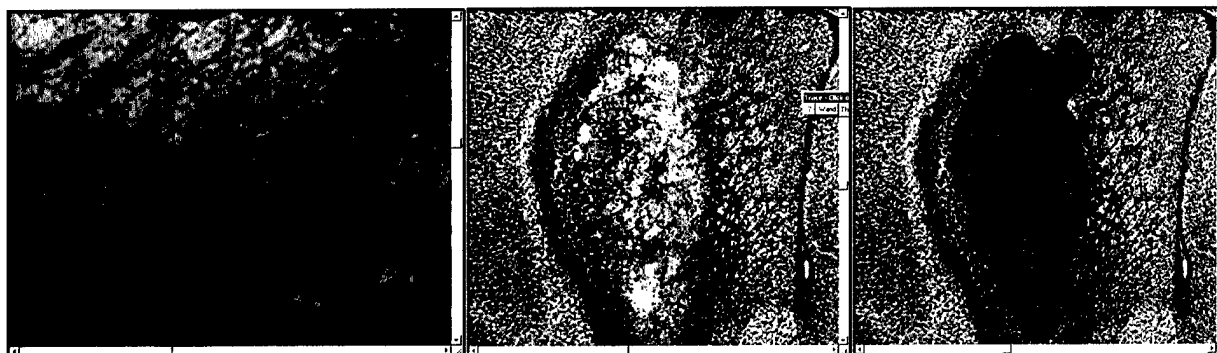
Reports published recently indicate that neurotoxins other than MPTP may also lead to aggregate formation (8, 9). In our hands, paraquat produces a similar degree of nigral degeneration as MPTP but with more clear-cut aggregate formation. We tested paraquat and the proteasomal inhibitors lactacystin and epoxomicin (10) because of the potential role of proteasomal dysfunction in aggregate formation. Preliminary studies suggested that both lactacystin and epoxomicin reduce sensitivity to MPTP toxicity but, paradoxically, there is a suggestion that synuclein immunoreactivity may be increased. Further studies will be needed to make a more definitive statement on this issue.

We performed a series of studies using alpha synuclein knockout mice, an important new animal model that was not available when this proposal was initially funded (11). With these animals we can directly test the role of synuclein in aggregate formation and the role of synuclein in MPTP neurotoxicity. Baseline histological studies show that they are no different from wild type (11). We found that alpha synuclein deficient mice are resistant to systemic MPTP-induced degeneration of

dopaminergic neurons. These effects were not due to alterations in MPTP processing, MPP⁺ uptake or vesicular transport. There was reduced generation of reactive oxygen species in alpha synuclein deficient mice following administration of 3-NP, and reduced histopathologic evidence of oxidative damage following MPTP, 3-NP and malonate. Cross-sectional area of striatal lesions produced by local injections of malonate was defined on spatially calibrated digital images of the striatum taken at 2 X at the level of the injection needle track. The circumference of intrastriatal injections were traced and the area then measured and recorded, as shown below:



All Error Bars = 1 Unit SE



A

B

C



D

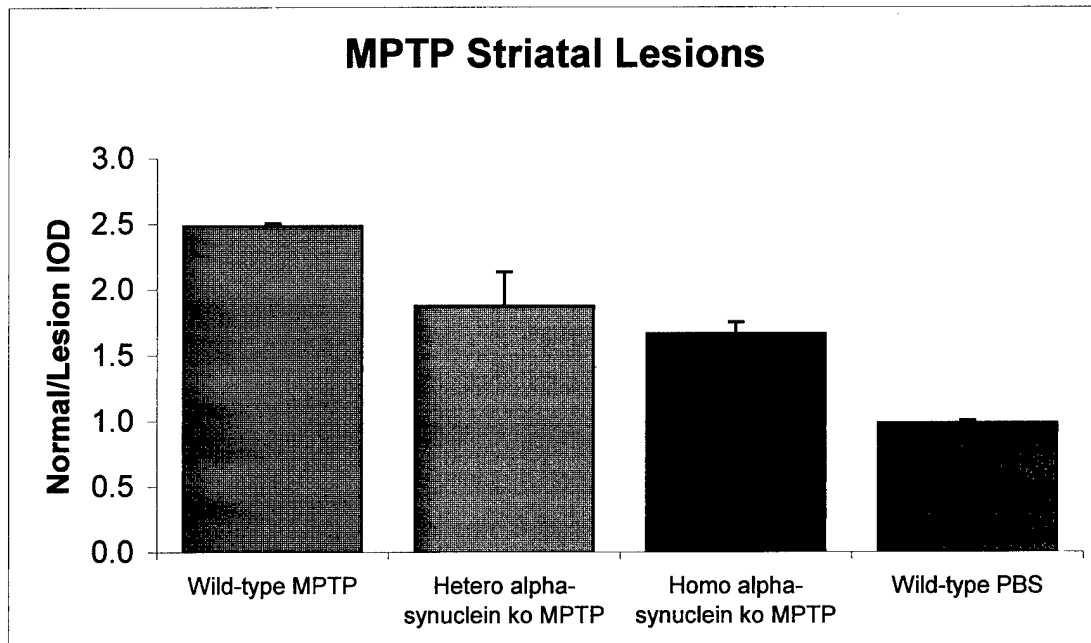


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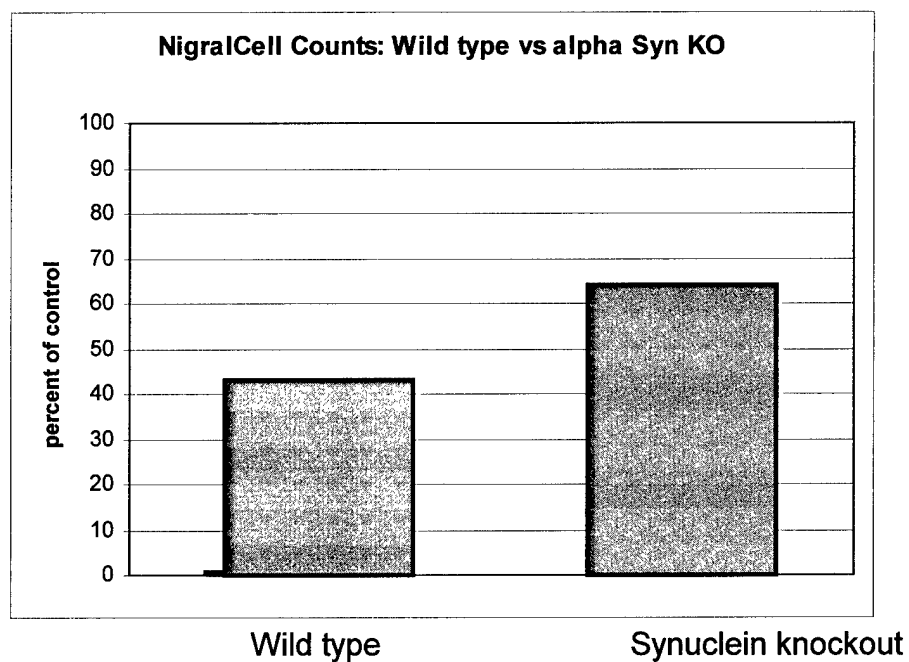
A. Dopamine Transporter (DAT) stained substantia nigra from a 3 nitropropionic acid (3NP) treated animal. This panel illustrates how the cell profiles are traced. Arrow shows a newly traced cell. Red profiles have been identified and measured. B & C. Nissl stained striatum from an animal treated with malonate. The plates show the lesion as it is traced (B) and after it has been identified and measured (C). D. DAT stained photomicrograph of the striatum of an animal that was treated with MPTP. The green outline (arrow) shows the area of interest (AOI) as it is placed over tissue that is adjacent to the one already measured within the lesion

These findings implicate alpha synuclein as a modulator of oxidative damage, which has been implicated in neuronal death produced by MPTP and other mitochondrial toxins.

The integrated optical density of DAT immunoreactivity in the striatum after MPTP administration showed a dose-dependent relationship to alpha synuclein expression as shown in this graph:



This protective effect was also found when we counted DAT immunoreactive neurons in the substantia nigra of MPTP treated mice plotted as a percentage of uninjected control values



Nigral cell counts were reduced by 35% in synuclein knockout mice compared To A 55% reduction in wild type animals.

Key Research Accomplishments:

The first four accomplishments listed below were generated based on the first series of experiments as delineated in the approved Statement of Work. These experiments were designed to define changes in the distribution and morphology of alpha synuclein immunoreactivity produced by systemic treatment of MPTP in mice. The remaining accomplishments were generated by the second series of experiments, which were focused on defining spatial and temporal relationships between synuclein aggregation and oxidative injury at the cellular level. Patterns of cell death and apoptosis associated with MPTP toxicity were determined and related to the changes in synuclein and oxidative damage, as stated in the approved statement of work.

- 1) Acute and chronic MPTP treatment, which cause nigral dopaminergic neurons to degenerate, are associated with the displacement of alpha synuclein from its normal synaptic location into neuronal cell bodies. Neuronal degeneration is evident with DAT and calbindin immunocytochemistry and glial reaction is evident with GFAP immunocytochemistry.
- 2) The redistribution of synuclein is associated with increased ubiquitin immunoreactivity and increased levels of oxidative markers in the substantia nigra.
- 3) The redistribution of synuclein does not appear to be associated with changes in distribution of synaptophysin or neurofilament proteins.
- 4) The redistribution of alpha synuclein from its normal synaptic location into neuronal cell bodies caused by MPTP treatment was confirmed in three mouse strains (C57BL/6, B6CBA and B6SJL) using four different anti-alpha synuclein antibodies.
- 5) The redistribution of alpha synuclein was associated with increased ubiquitin immunoreactivity and increased levels of malondialdehyde (MDA), 3-nitrotyrosine (NT) and 8-hydroxy-deoxyguanosine immunoreactivity in the substantia nigra, but changes in distribution of synaptophysin or

- neurofilament protein do not occur. This suggests that a general disruption of neuronal polarity affecting synapses or dendrites is not produced by MPTP toxicity.
- 6) The localization of activated caspase 3 immunoreactivity to neurons in the substantia nigra suggests that apoptotic pathways are activated by MPTP.
- 7) Time course studies suggest that alpha synuclein aggregation is a relatively late phenomenon after MPTP treatment. Alpha synuclein immunoreactive aggregates are detected at 7-10 days post MPTP exposure but not 4 days post exposure.
- 8) Double labeling studies show evidence of oxidative damage 3 days prior to the development of increased alpha synuclein immunoreactivity on day 7 post exposure. Oxidative markers were not increased 24 hours post MPTP exposure.
- 9) Paraquat poisoning induces nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by MPTP.
- 10) The proteasomal inhibitors lactacystin and epoxomicin protect mice from the neurotoxic effects of MPTP but may be paradoxically associated with increased alpha synuclein aggregation in nigral neurons.
- 11) Alpha synuclein knockout mice resist the neurotoxic effects of MPTP and mitochondrial toxins. Quantitative analysis shows that lesions produced by intrastriatal malonate are smaller in synuclein knockout mice and that MPTP toxicity is reduced in heterozygous synuclein knockout (+/-) mice and further reduced in homozygous synuclein knockouts (-/-).

Reportable Outcomes

- 1) A paper and abstracts have been published and results have been presented at national meetings. A manuscript has been prepared for submission.
- 2) A database of histological materials has been enlarged and a large number of specimens have been added to our tissue bank and catalogued. These will be available for future research.

- 3) Four postdoctoral fellows and four technicians have been trained in surgical and histological procedures and have gained experience in the laboratory supported by this award

Conclusions

MPTP treated mice develop increased alpha synuclein immunoreactivity in neurons that are degenerating in the substantia nigra 7-10 days after MPTP administration. This is true in both the acute and chronic MPTP models in three different strains of mice using four different synuclein antibodies. Degenerating neurons are identified using dopamine transporter and calbindin immunocytochemistry and glial reaction is identified with glial acidic fibrillary protein. Synaptophysin and neurofilament immunoreactivity are not altered suggesting that MPTP toxicity may have a specific effect on alpha synuclein. The neurodegenerative process is associated with increased levels of oxidative markers for DNA, protein and lipids as indicated by immunocytochemistry for 8-hydroxydeoxyguanosine, 3-nitrotyrosine and malondialdehyde respectively. Ubiquitin immunoreactivity is also prominent in degenerating neurons. In addition activated caspase 3 is detected in degenerating neurons suggesting that apoptotic pathways are activated. Our observations validate the MPTP model of PD by demonstrating that MPTP causes increased synuclein staining in degenerating neurons even though classical Lewy bodies are not produced.

In contrast to increased alpha synuclein immunoreactivity, which develops 7-10 days after toxin administration, oxidative injury is present in dopaminergic neurons 4 days post toxin administration. Double staining studies show that oxidative markers are persistently increased in neurons that show increased alpha synuclein staining. Paraquat treatment causes nigral degeneration and alpha synuclein aggregation that is more prominent than that produced by MPTP. The proteasomal inhibitors lactacystin and epoxomicin protect mice from the neurotoxic effects of MPTP but may paradoxically increase alpha synuclein aggregation. Alpha synuclein knockout mice resist the neurotoxic effects of MPTP and other mitochondrial toxins including malonate, 3-nitropropionic acid and paraquat.

In summary, our studies show that MPTP-induced oxidative injury precedes increased neuronal alpha synuclein staining and suggest that alpha synuclein and abnormal proteasomal function could contribute to neurotoxin and PD-related neuronal cell death. We believe that these findings provide new insights into the pathogenesis of neuronal degeneration induced by neurotoxins and suggest that therapeutic strategies targeted at reducing synuclein expression may be a novel approach to the treatment of PD.

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A. Journal article and Manuscript

- 1) Kowall NW, Hantraye P, Brouillet E, Beal MF, McKee AC and Ferrante RJ. MPTP induces alpha-synuclein aggregation in the substantia nigra of baboons. *Neuroreport* 2000;11(1):211-213.
- 2) Klivenyi P, Ferrante RJ, Giardian G, Yang L, Albers D, Kowall NW, Abelovich A, Beal MF. *Mice Lacking Alpha-Synuclein Are Resistant To Mitochondrial Toxins, in preparation*

B. Abstracts

- 1) Mahoney SC, Ferrante RJ, Dedeoglu A, McKee AC, Kowall NW. Alpha Synuclein is selectively redistributed in the substantia nigra of MPTP treated mice. *J Neuropathology Exptl Neurology* 2001; 60:549.

- 2) Kowall NW. Alpha Synuclein in a neurotoxic model of Parkinson's disease. Workshop on DoD-Sponsored Parkinson's Related Research. March 2001, page 73.

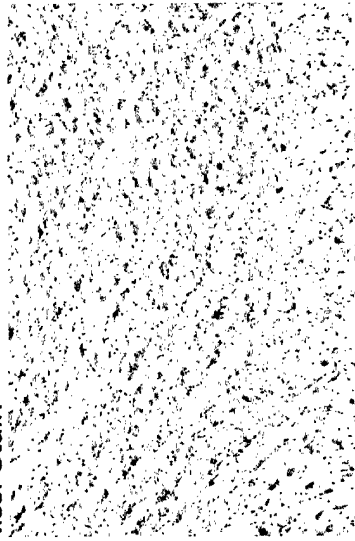
LIST OF PERSONNEL

Neil W. Kowall, MD:	Principal Investigator
Robert J. Ferrante, PhD:	Investigator
Peter Morin, MD, PhD:	Post-Doc
Nazar Quereshi, MD:	Post-Doc
Donald Siwek, PhD:	Post-Doc
Catherine O'Malley, PhD:	Post-Doc
Megan Lavoie, BS:	Lab Technician
Sean Mahoney, BS:	Lab Technician
Kimberly Crawford, BS:	Data Entry/Lab Technician
Karthik Venkatesh:	Data Entry/Lab Technician

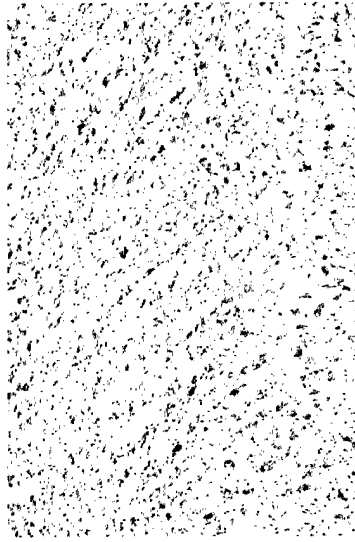
Figure 1. Histology of the substantia nigra (low power)

Control

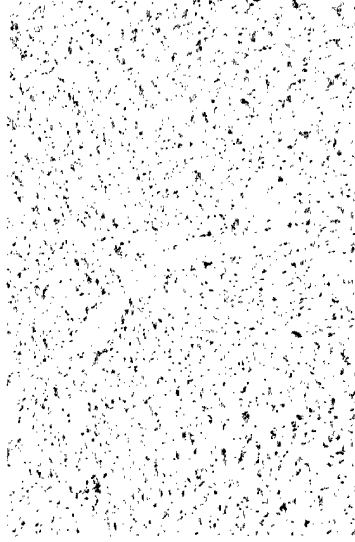
Nissl Stain



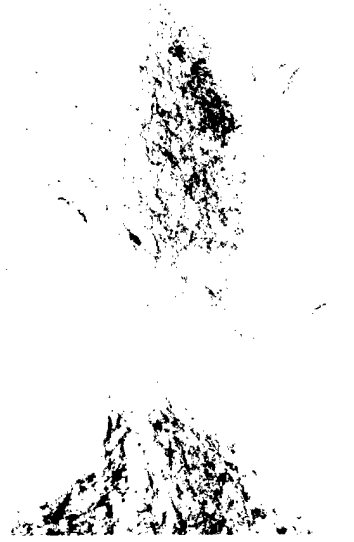
Acute MPTP



Chronic MPTP



Dopamine Transporter



Glial Fibrillary Acidic Protein

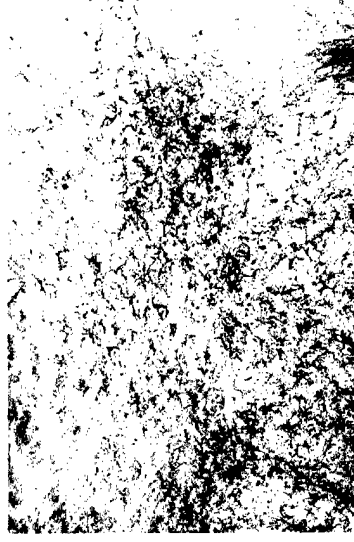
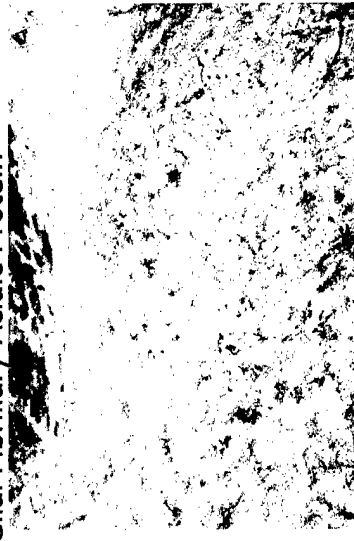


Figure 1: Histology of the Substantia Nigra. A series of photomicrographs that show the effects of acute vs chronic MPTP administration. Both chronic and acute MPTP administration cause neuronal loss and increase glial reaction in the substantia nigra.

Figure 2a. Histology of the Substantia Nigra (High Power) I

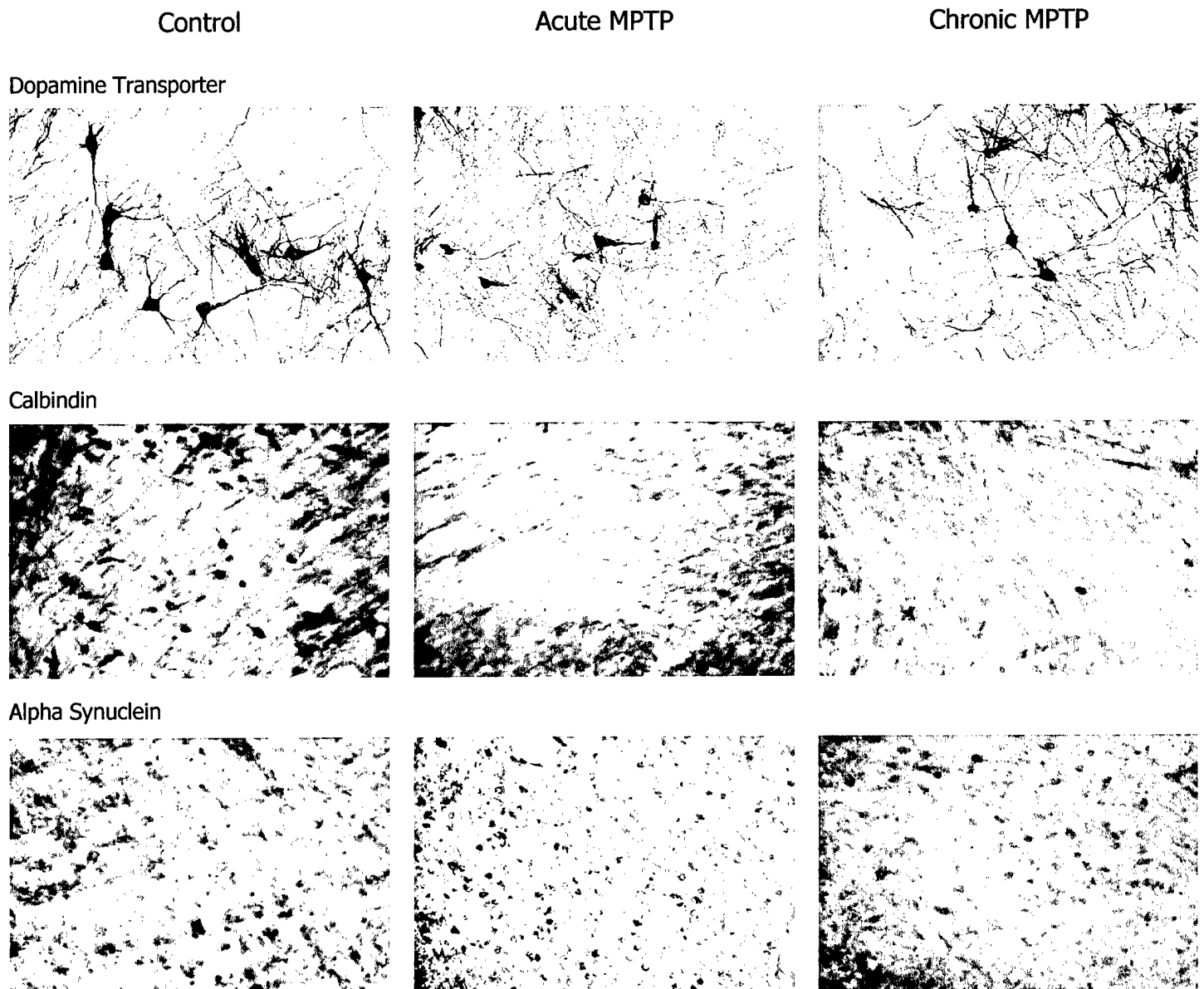


Figure 2a. High power photomicrographs showing the effects of MPTP in the substantia nigra. These images show striatal neuronal degeneration caused by MPTP. Calbindin immunoreactivity is depleted, but alpha synuclein immunoreactivity is increased in both acute and chronic MPTP administration.

Figure 2b: Histology of the Substantia Nigra (High Power) II

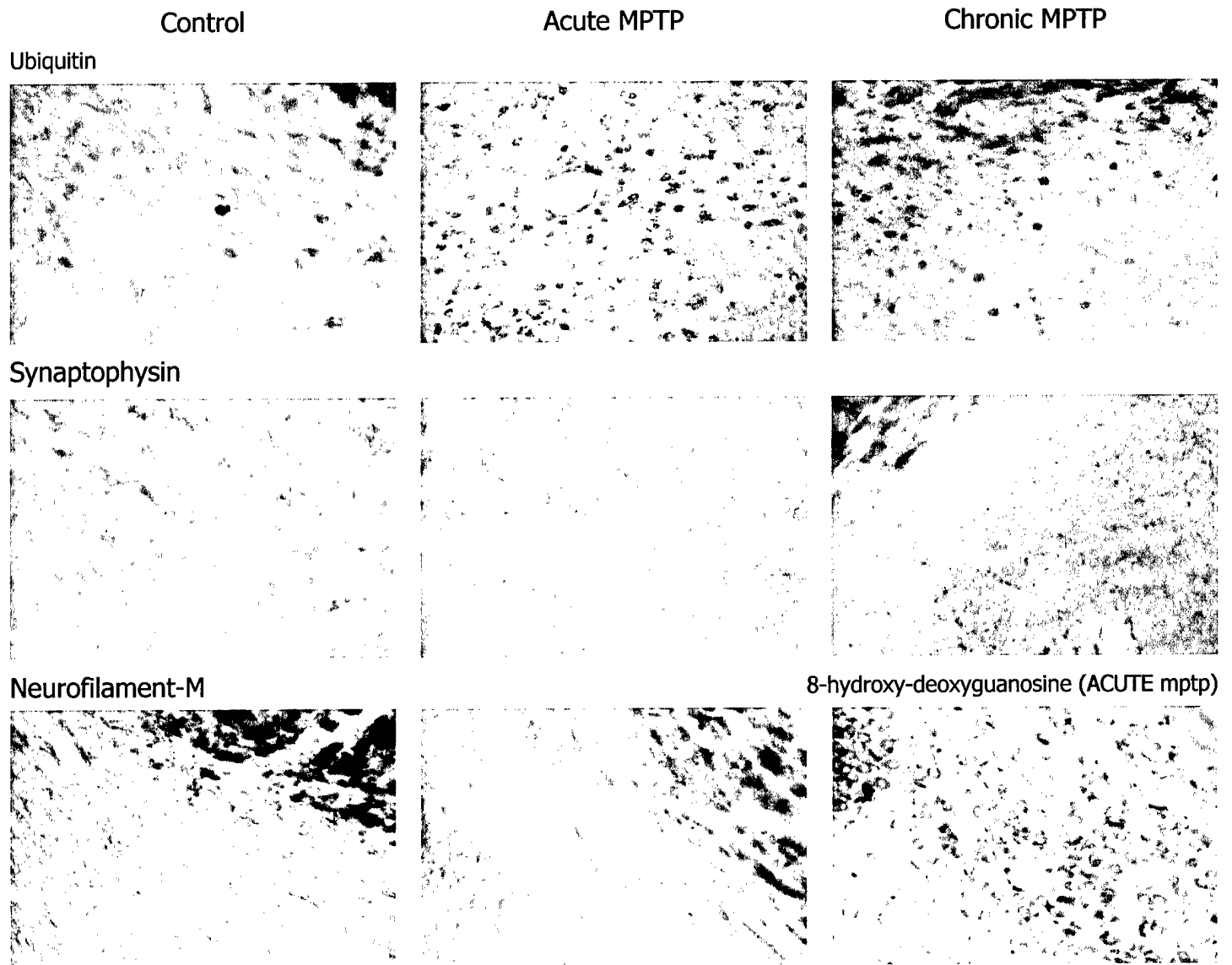


Figure 2b. High power photomicrographs showing the effects of MPTP in the substantia nigra. These images show striatal neuronal degeneration caused by MPTP. Ubiquitin immunoreactivity is increased after both acute and chronic administration of MPTP. In contrast, synaptophysin and neurofilament-M show no clear changes. 8-OHDG (lower right) is increased reflecting oxidative changes.

Figure 3: DOPAMINE TRANSPORTER

Striatum - Control

Striatum - Acute MPTP

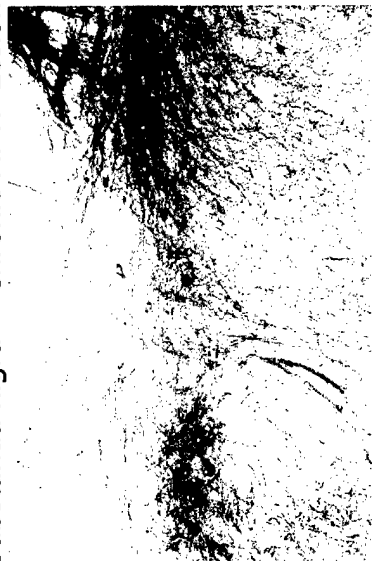
Striatum - Chronic MPTP



Substantia nigra - Control

Substantia nigra - Acute MPTP lesion

Substantia nigra - Chronic MPTP Lesion



Substantia nigra (high power) Control

Substantia nigra - Acute MPTP lesion

Substantia nigra - Chronic MPTP Lesion

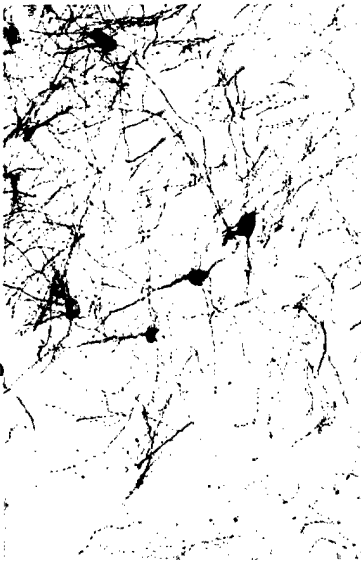
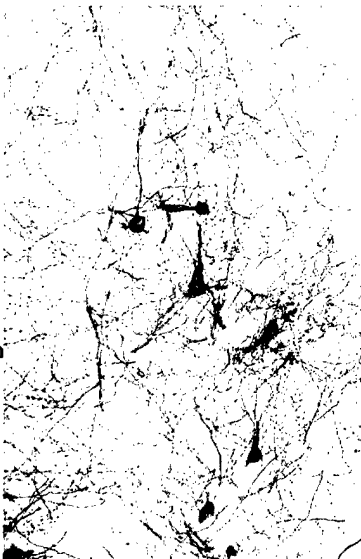
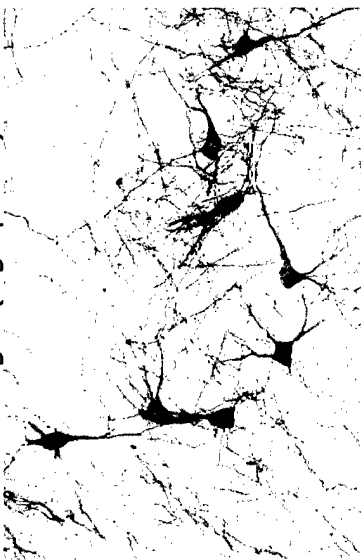
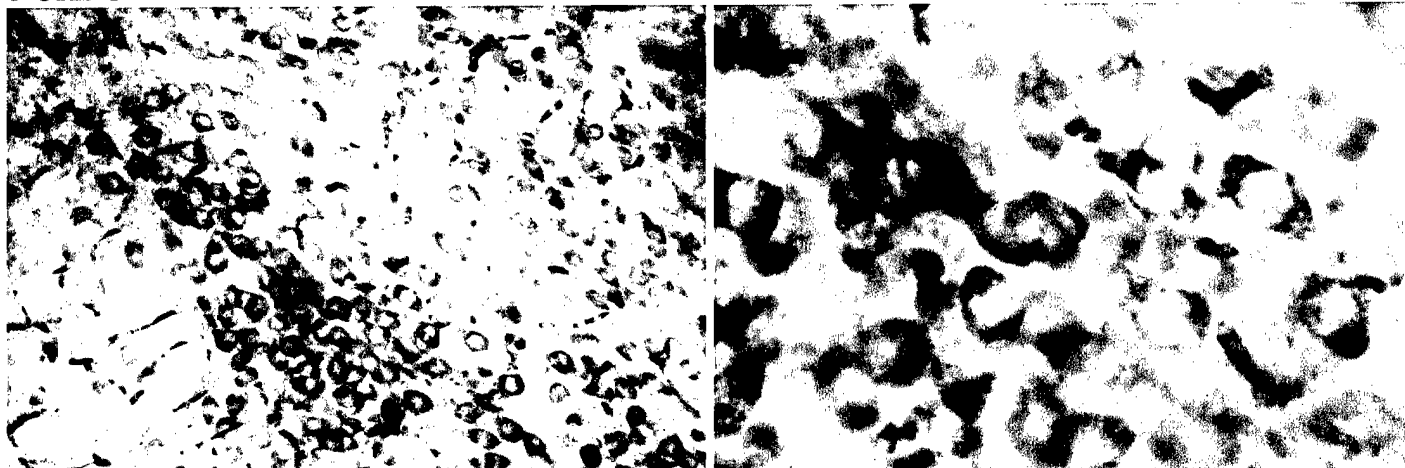


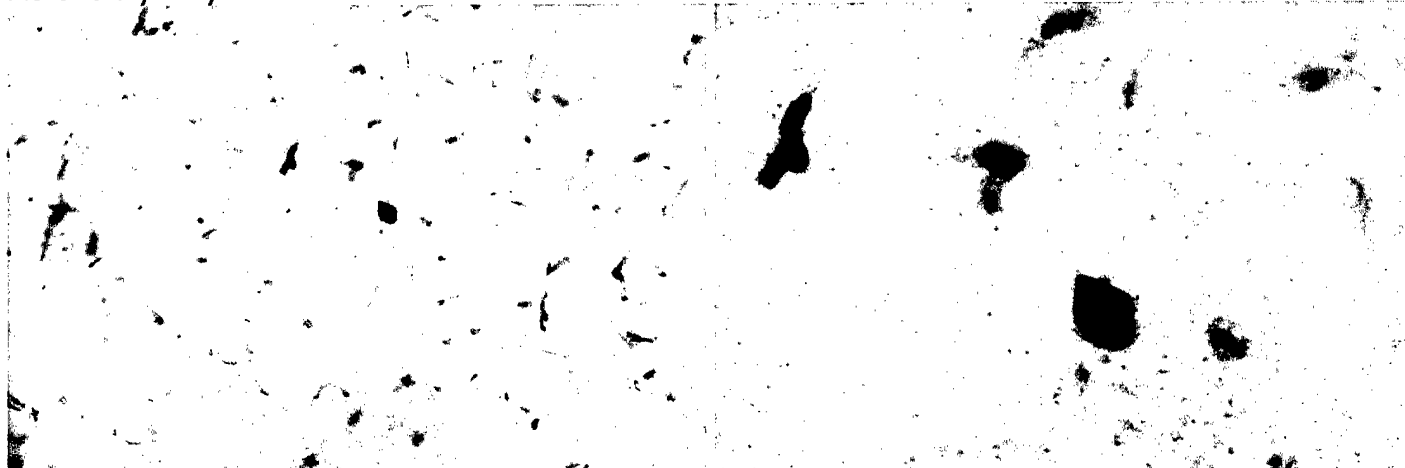
Figure 3. Color photomicrographs matching black and white images found in the body of the report showing striatal dopamine transporter depletion and neuronal loss in the substantia nigra.

**FIGURE 4: OXIDATIVE MARKERS IN THE MPTP-TREATED MOUSE SUBSTANTIA NIGRA
(LOW AND HIGH POWER)**

8-OHDG



Malondialdehyde



Nitrotyrosine

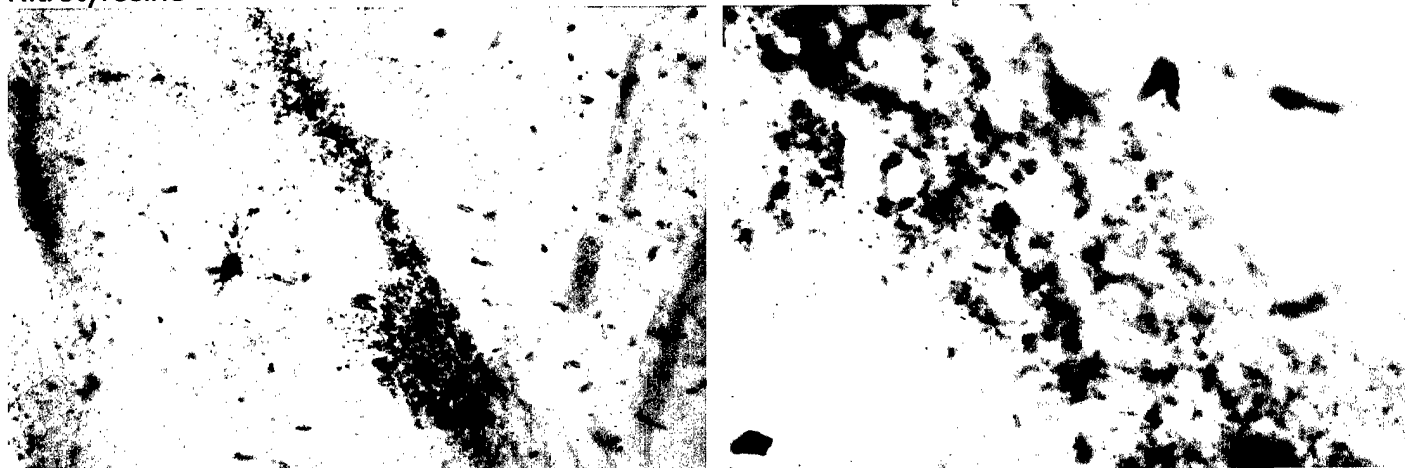
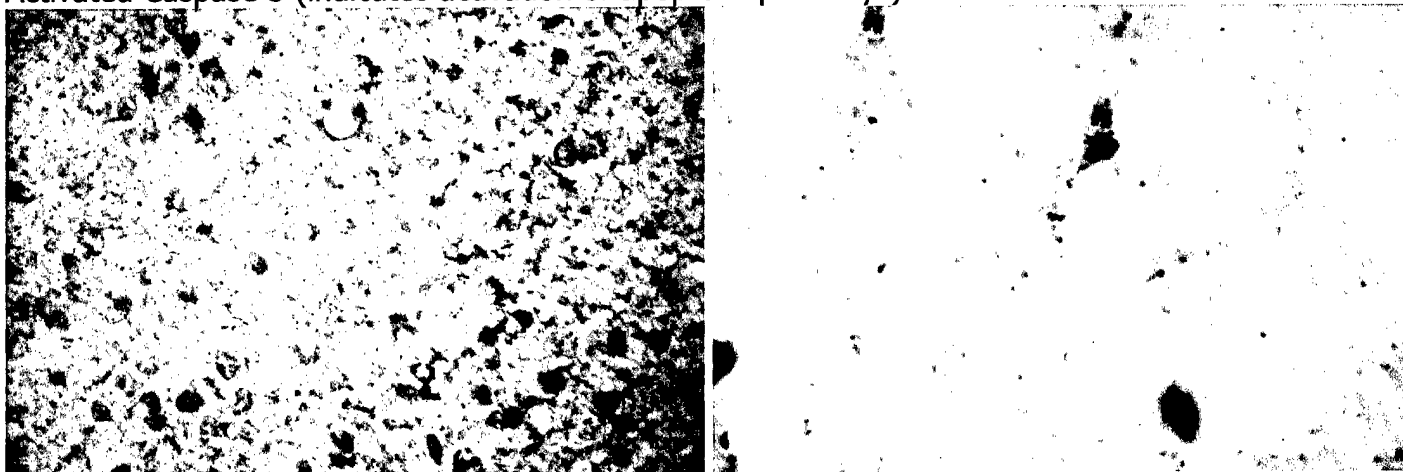


Figure 4. Oxidative marker studies indicate that DNA, Lipids and proteins are oxidized after MPTP administration.

**FIGURE 5: APOPTOSIS AND STRESS MARKERS IN MPTP-TREATED MOUSE SUBSTANTIA NIGRA
(LOW AND HIGH POWER)**

Activated Caspase 3 (indicates activation of apoptotic pathways)



Ubiquitin

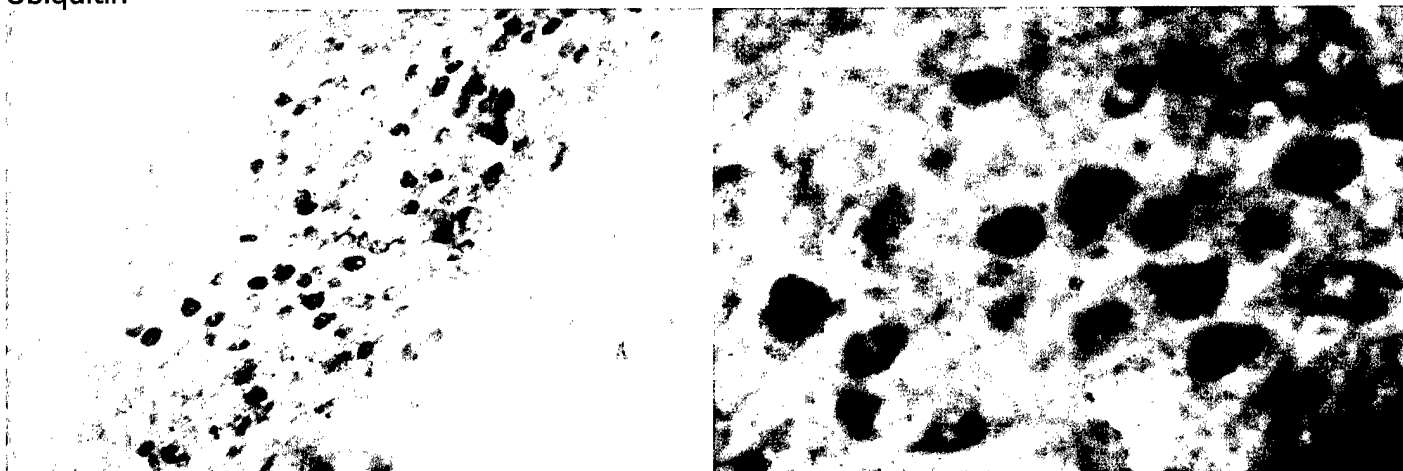
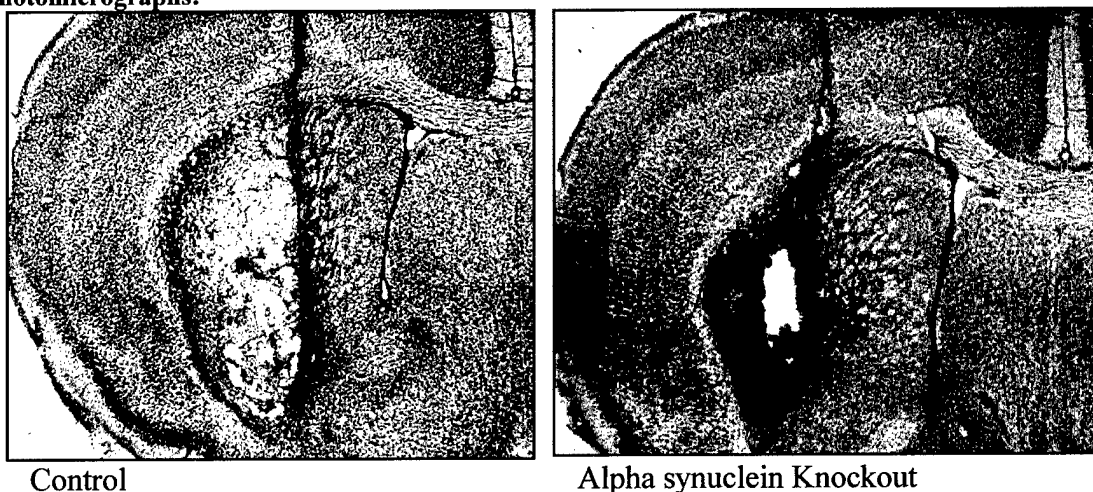


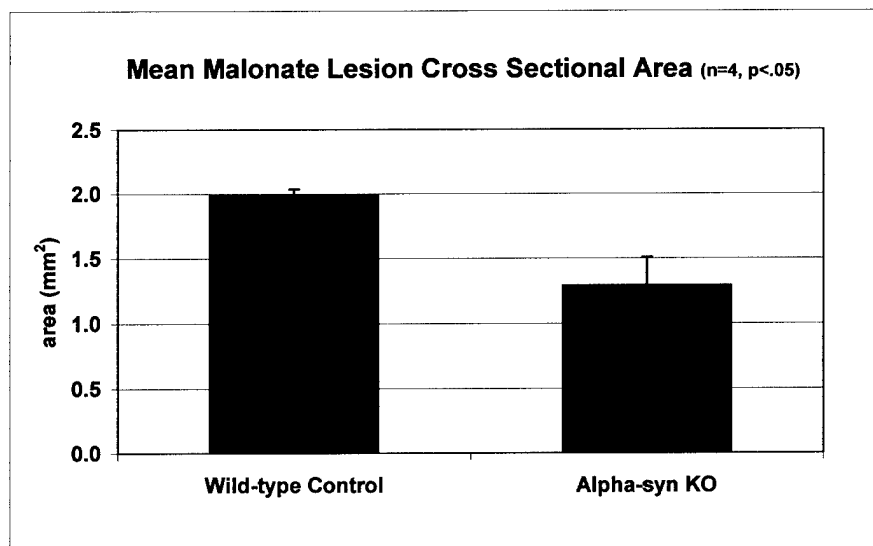
Figure 5. MPTP treatment induces activated Caspase 3 expression and increased Ubiquitin reactivity.

Figure 6: INTRASTRIATAL INJECTIONS OF THE MITOCHONDRIAL TOXIN MALONATE.

Photomicrographs:



Graph



Data table. Cross-sectional area (μm^2)

Wild-type control	Alpha-syn KO
1916512	1374455
2021285	1857957
2111630	1091274
1898049	835645

Single factor ANOVA

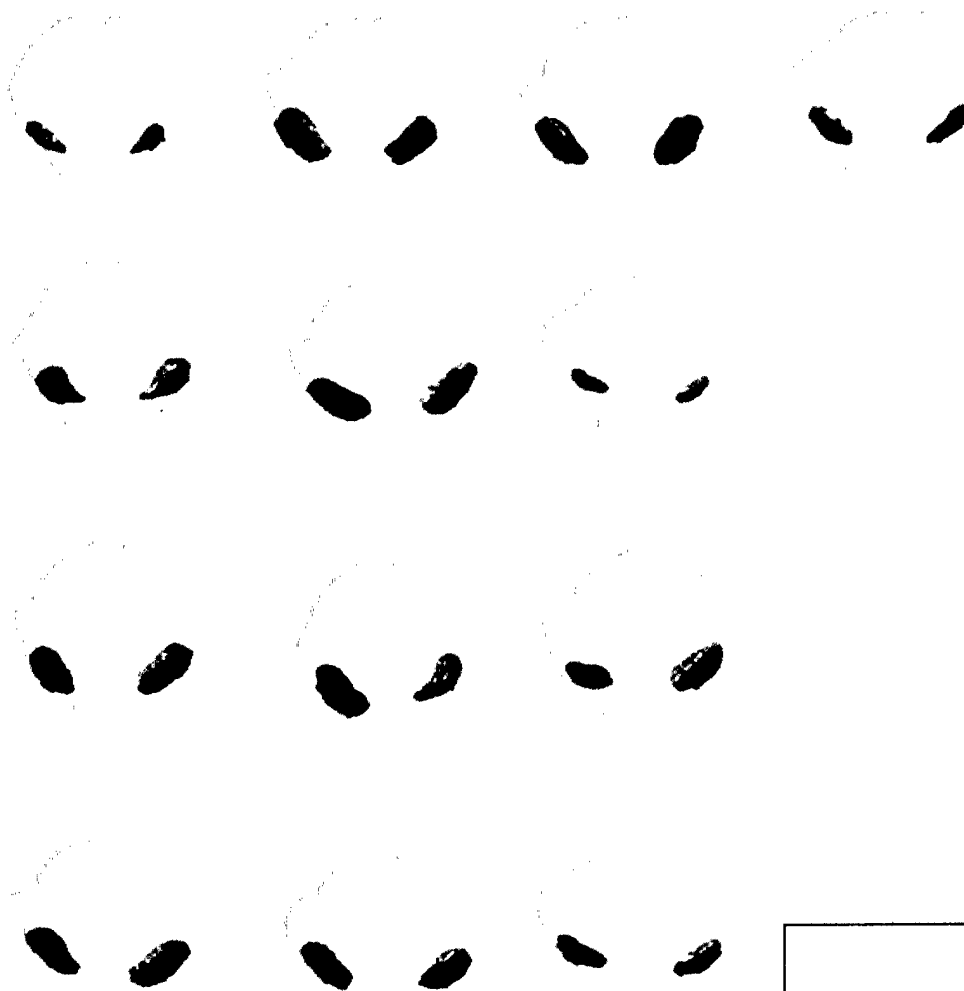
SUMMARY

Groups	Count	Sum	Average	Variance
Wild-type Control	4	7947475.8	1986869	9.863E+09
Alpha-Syn KO	4	5159330.69	1289833	1.919E+11

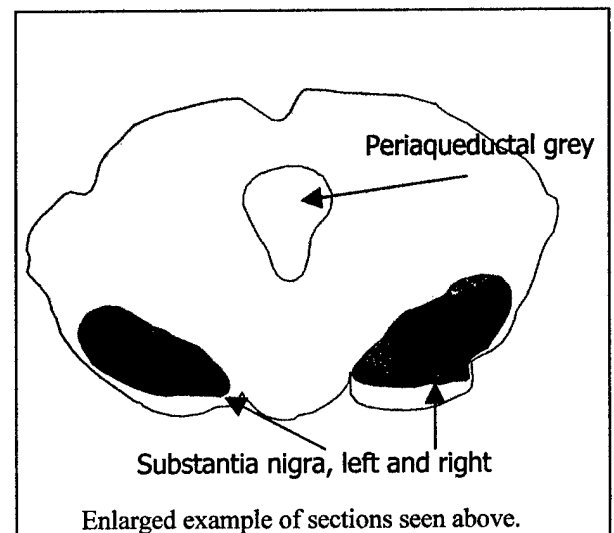
ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.72E+11	1	9.72E+11	9.6332559	0.021016	5.987374152
Within Groups	6.05E+11	6	1.01E+11			
Total	1.58E+12	7				

Figure 7a. TOPOGRAPHIC DISTRIBUTION OF DOPAMINE TRANSPORTER IMMUNOPOSITIVE NEURONS IN THE SUBSTANTIA NIGRA I.



Wild-type PBS injected

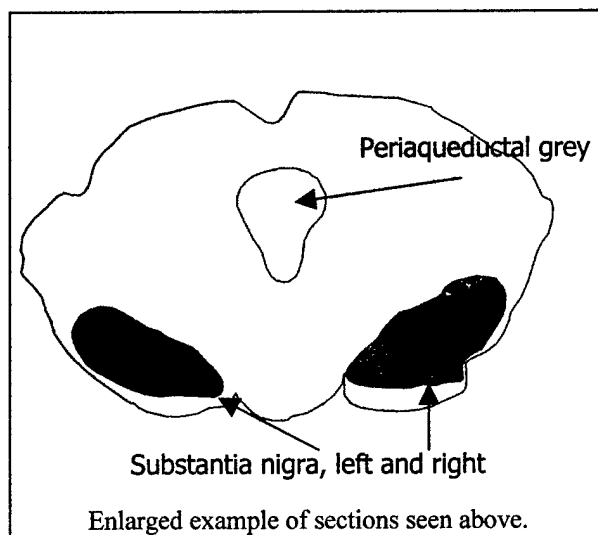


Neurolucida charting of dopamine transporter immunopositive neurons in serial sections through the brainstem. Sections were cut at 50um thickness and are 300 um apart. Each row shows data from individual animals. Data analyses results are depicted in figure 9. The gray profiles are sections of brainstem, the yellow profiles indicate the region of periaqueductal gray matter, the two black areas represent the substantia nigra and the colored dots represent identified DAT positive neurons.

Figure 7b. TOPOGRAPHIC DISTRIBUTION OF DOPAMINE TRANSPORTER IMMUNOPOSITIVE NEURONS IN THE SUBSTANTIA NIGRA II.

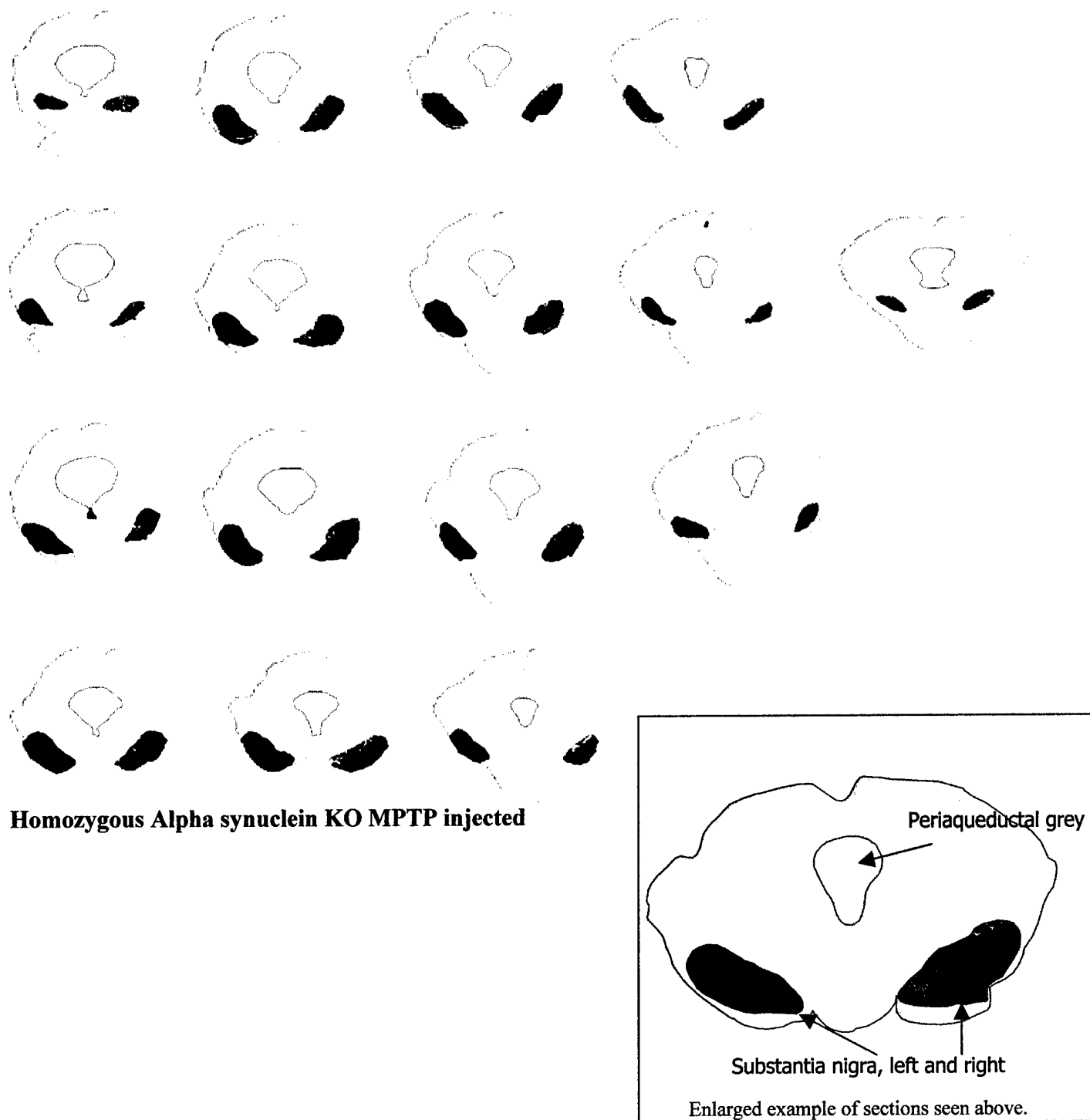


Wild-type MPTP injected



Neurolucida charting of dopamine transporter immunopositive neurons in serial sections through the brainstem. Sections were cut at 50um thickness and are 300 um apart. Each row shows data from individual animals. Data analyses results are depicted in figure 9. The gray profiles are sections of brainstem, the yellow profiles indicate the region of periaqueductal gray matter, the two black areas represent the substantia nigra and the colored dots represent identified DAT positive neurons.

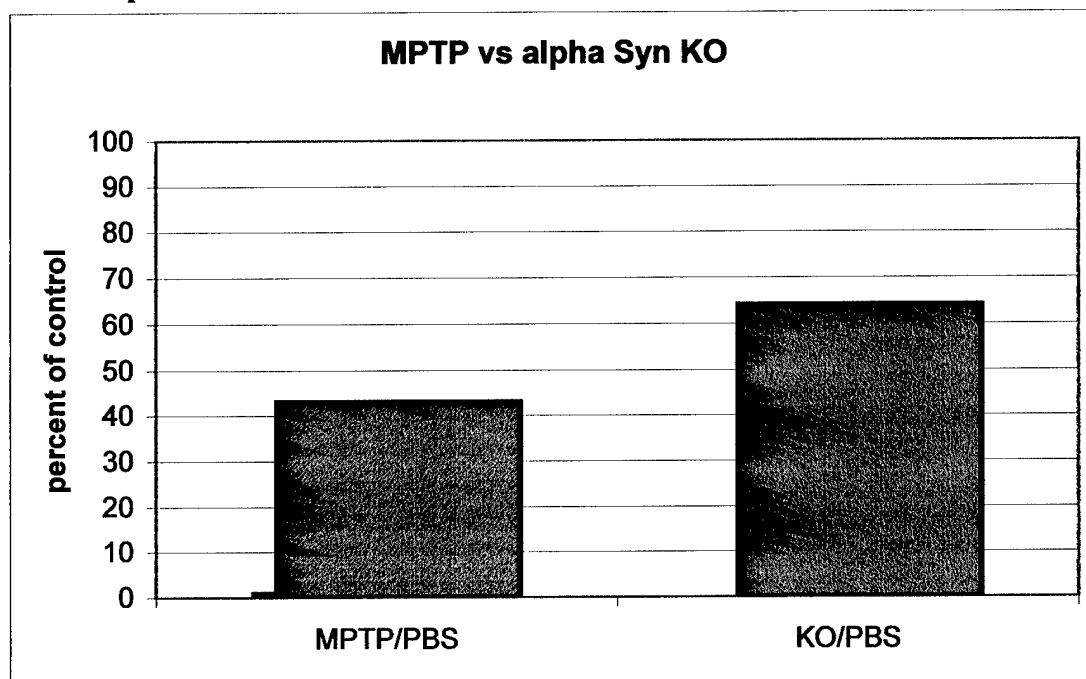
Figure 7c. TOPOGRAPHIC DISTRIBUTION OF DOPAMINE TRANSPORTER IMMUNOPOSITIVE NEURONS IN THE SUBSTANTIA NIGRA III.



Neurolucida charting of dopamine transporter immunopositive neurons in serial sections through the brainstem. Sections were cut at 50um thickness and are 300 um apart. Each row shows data from individual animals. Data analyses results are depicted in figure 9. The gray profiles are sections of brainstem, the yellow profiles indicate the region of periaqueductal gray matter, the two black areas represent the substantia nigra and the colored dots represent identified DAT positive neurons.

Figure 8: SUBSTANTIA NIGRA CELL COUNTS IN MPTP INJECTION STUDIES

Data Graph:



Data Table:

Number of DAT+ cells in the Substantia Nigra in each of three treatment groups

Case	MPTP	KO	PBS
1	244	453	804
2	383	411	616
3	453	330	675
4	85	540	615
Case averages	291.25	433.5	677.5

ANOVA:

SUMMARY

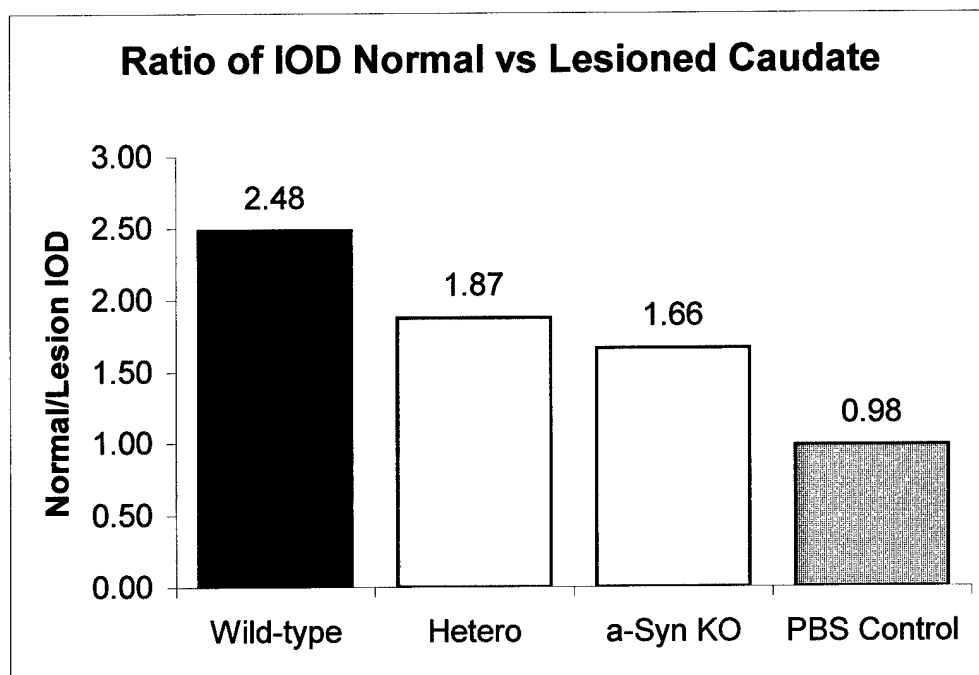
Groups	Count	Sum	Average	Variance
MPTP	7	2030	290	13987
KO	4	1734	433.5	7647
PBS	4	2710	677.5	7899

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	382235.6	2	191117.8	17.56597	0.000272	3.885290312
Within Groups	130560	12	10880			
Total	512795.6	14				

Figure 9a: INTEGRATED OPTICAL DENSITY MEASUREMENTS IN MPTP STUDIES.

Data Graph:



In the above chart, each column represents the average integrated optical density (IOD) of DAT immunoreactivity in the caudate nucleus of animals in each of 4 treatment groups. The Y-axis is a calculated value obtained by dividing IOD of unaffected tissue by the IOD of caudate tissue lesioned by administration of MPTP.

Data Table:

PBS Control	Wild Type	a-Syn KO	Het.
1.00	1.63	1.36	1.54
0.98	2.49	1.42	1.18
0.94	2.33	1.65	1.27
0.92	1.20	1.92	2.57
0.93	1.64	1.80	2.57
0.93	1.98	1.81	2.12
1.04	2.56		
1.02	2.28		
1.08	2.34		
	2.44		
	1.56		
	4.29		
	5.32		
	3.21		
	1.82		
	2.91		
	2.23		

FIGURE 9B: SINGLE FACTOR ANOVA STATISTICS FOR INTEGRATED OPTICAL DENSITY MEASUREMENTS IN MPTP STUDIES

Anova: Single Factor				
SUMMARY				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
PBS	9	8.835629	0.981737	0.003362
MPTP	17	42.22809	2.484006	1.048005
KO	6	9.965146	1.660858	0.050705
HET	6	11.24246	1.873744	0.399958

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	13.734	3	4.578023	8.171481	0.000313	2.882601
Within Groups	19.048	34	0.560244			
Total	32.782	37				

Figure 9b. ANOVA Tables for data obtained from IOD measurements in the Caudate nucleus of wild-type controls, heterozygous alpha synuclein KO, homozygous alpha synuclein KO and PBS injected alpha synuclein KO animals.